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(54) Title: PROCESS FOR ISOLATING NUCLEIC ACID FROM GRAM POSITIVE MICROORGANISMS

(57) Abstract

The invention relates to a process, a combination of means for isolating nucleic acid from a nucleic acid-containing starting material and a test kit in order to amplify the nucleic acid obtained by said process. More in particular, the invention relates to a process and a kit for isolating nucleic acid from nucleic acid-containing gram positive microorganisms found in materials such as food, whole blood, blood serum, urine, feces, cell cultures and the like.

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Process for Isolating Nucleic Acid
From Gram Positive Microorganisms

The invention relates both to a process and a combination of means for isolating nucleic acid from a
5   nucleic acid-containing starting material as well as a testkit in order to amplify the nucleic acid obtained by said process. More in particular, the invention relates to a process and a kit for isolating nucleic acid from a nucleic acid-containing biological material, such as whole
10   blood, blood serum, buffy coat (the crusta phlogistica or leukocyte fraction of blood), urine, feces, liquor cerebrospinalis, sperm, saliva, tissues, cell cultures and the like. Nucleic acid as isolated from above-mentioned biological material can also comprise the endogenous
15   nucleic acid from the organism from which the sample is derived and any foreign (viral, fungal, bacterial or parasitic) nucleic acid.

Known methods of isolating nucleic acid (NA) from complex starting materials like whole blood, blood serum,
20   urine or feces usually comprise lysis of biological material by a detergent in the presence of protein degrading enzymes, followed by several extractions with organic solvents, e.g., phenol and/or chloroform, ethanol precipitation and dialysis of the nucleic acids. These
25   known methods of, e.g., isolating (double-stranded) DNA from clinical material are very laborious and time-consuming. The relatively large number of steps required to purify NA from such starting materials increase the risk of transmission of NA from sample to sample in the
30   simultaneous processing of several clinical samples. When the NA is isolated for the subsequent detection of the presence of NA of, e.g., a pathogen (e.g., a virus or a bacterium) by means of a nucleic acid amplification method

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for example the utmost sensitive polymerase-chain-reaction (PCR, Saiki et al, Science 230, 1985, 1350), the increased risk of such a transmission of NA between different samples which causes false positive results is a serious drawback.

5   An example of such a known method sensitive to contamination is the procedure described in Analytical Biochemistry 162, 1987, 156 for isolating total RNA from tissues and cell cultures. According to this method the RNA is subjected to a single extraction with an acid
10   guanidinium thiocyanate-phenol-chloroform mixture from the biological starting material. After phase separation the RNA can be recovered in useful condition within 4 hours by further processing the aqueous phase.

In Analytical Biochemistry 162, 1987, 463, there is
15   described a procedure for isolating DNA from tissues and cell lines, in which the cells are dispersed in a guanidine hydrochloride-containing buffer and ethanol precipitated. With this known method sensitive to contamination a useful NA product can also be isolated within a few hours after
20   further processing the separated DNA.

These known procedures, however, cannot be used successfully in complex starting materials, e.g., whole blood and blood serum.

It is an object of the invention to provide a process
25   which removes the drawbacks of the known processes.

More in particular, it is an object of the invention to provide a process with which nucleic acid (i.e. DNA and/or RNA) can be isolated immediately (without pretreatments) from complex starting materials, such as
30   different types of biological materials, in an unprecedentedly rapid, simple and reproducible manner in such undamaged conditions and high purity that it can then be used as a reagent in molecular biological reactions.

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It is a further object of the invention to provide a
process which differs from the known processes by a low
risk of contamination as compared with other samples and
persons, i.e. enables simultaneous processing of several
5   clinical samples at a minimum risk of transmission of NA
between different samples, and also means a lowest
possible risk of contagion of persons by viruses or
bacteria that may be present in the samples to be
processed.

10   These objects are realized according to the invention
by a process for isolating nucleic acid from a nucleic
acid-containing starting material, characterized by mixing
the starting material, characterized by mixing the starting
material with a chaotropic substance and a nucleic acid
15 binding solid phase, separating the solid phase with the
nucleic acid bound thereto from the liquid, whereafter thus
obtained solid phase-nucleic acid complexes were washed,
and if required the nucleic acid was eluted from said
complexes.

20   Although in a wide sense the invention is applicable
to any nucleic acid-containing starting material, including
foods and allied products, vaccines and milk infected with
a virus or a bacterium, the invention is particularly
applicable to a process in which the starting material
25 employed is a nucleic acid-containing biological material,
such as whole blood, blood serum, buffy coat, urine, feces,
liquor cerebrospinalis, sperm, saliva, tissues and cell
cultures (such as mammalian cell cultures and bacterial
cultures).   Of course, the process according to the
30 invention is also applicable to relatively pure input
materials, such as the product of a PCR or the product to
be purified further of another process for recovering
nucleic acids.   However, some types of nucleic
acid-containing biological materials, such as vegetable

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material, some gram-positive bacteria and some yeasts and moulds, cannot immediately function as an input material in the process according to the present invention, because owing to their special cell wall structure they do not lyse

5   into a chaotropic substance. Therefore, such starting materials require a pretreatment rendering the cells accessible, e.g., a preceding cell lysis, after which the resulting lysate can be subjected to the process according to the invention.

10   By nucleic acid (NA) is meant both DNA and RNA, both in any possible configuration, i.e. in the form of double-stranded (DS) nucleic acid, or in the form of single-stranded (ss) nucleic acid, or as a combination thereof (in part ds or ss).

15   Essential according to the invention is the use of a nucleic acid binding solid phase e.g. silica particles capable of binding the NA in the presence of a chaotropic substance. By silica are meant SiO_2 crystals and other forms of silicon oxide, such skeletons of diatoms built up

20   from SiO_2, amorphous silicon oxide and glass powder. Also alkylsilica, aluminium silicate (zeolite), activated silica with -NH2, latex particles, certain polymeric materials forming the inside wall of a cuvette or a microtiter plate, or filter materials for example consisting of

25   nitrocellulose are suitable as nucleic acid binding solid phase according to the invention.

For the matter using silica particles, it was known from PNAS 76, 1979, 615, that dsDNA in a highly concentrated solution of chaotropic salt NaI (sodium

30   iodide) can be released from agarose and can be bound to glass. This publication describes two procedures for isolating DNA from an agarose gel, both of which use in a first step an NaI solution to dissolve the agarose. In one procedure the DNA is precipitated in a second step with

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acetone, while according to the other procedure the DNA is bound in a second step to glass particles and is then eluted into an aqueous buffer. This method, however, is of no use to more complex starting materials, such as body fluids and other biological starting materials. In this article there is also no disclosure for an one-step procedure according to the invention.

It is recommendable according to the invention to use silica particles having a suitably selected particle size so that a high degree of purity of the bound and then eluted nucleic acid is immediately obtained from an impure starting material.

A preferred embodiment of the invention is characterized by using silica particles having a practical size ranging substantially between 0.05 and 500 μm. By the term "substantially" is meant that 80% or more, preferably more than 90%, of the silica particles are within the particle size range defined. In order to ensure easy processing of the bound NA, it is preferred that the silica particles employed have a particle size range substantially between 0.1 and 200 μm, while a process in which the silica particles employed have a particle size ranging substantially between 1 and 200 μm is most preferred. It is true that the NA-binding capacity of the silica particles is higher as the particles are smaller, but especially in the case of an NA-rich input material and in the case of relatively long NA molecules the use of extremely small silica particles will result in that the NA-silica complexes formed cannot be efficiently redispersed anymore. This means that the bound NA cannot be recovered from the complexes in a pure form. When human blood is used as an input material, this problem sometimes occurs if there is used a non-fractionated silica having particle sizes within the range of 0.2-10 μm. The

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formation of aggregates that cannot be redispersed anymore may be avoided by using a fractionated silica, the particle size of which is within the range of 1-10 μm. When an input material rich in cells is used, such as bacterial
5   cultures, it is found, however, that the use of such a coarse silica fraction is not sufficient to avoid the formation of hardly redispersible aggregates and optimum results are obtained if there is used an even coarser silica, such as a diatomaceous earth having particle sizes
10   within the range of 2-200 μm.

In another preferred embodiment the NA binding solid phase is in the form of a filter or even forms part of the vessel in which the sample with chaotropic substance is contained. The latter forms for the NA binding solid phase
15   eliminates the necessity of centrifugation or filtration for further sample processing and NA isolation.

According to the invention it is essential to use a chaotropic substance in addition to the above- mentioned nucleic acid binding solid phase such as silica particles.
20   By chaotropic substance is meant any substance capable of altering the secondary, tertiary and/or quaternary structure of proteins and nucleic acids, but leaving at least the primary structure intact. Examples thereof are guanidinium (iso)thiocyanate and guanidine hydrochloride.
25   Also sodium iodide, potassium iodide, sodium (iso)thiocyanate, urea or mutual combinations therewith are very suitable in combination with nucleic acid binding solid phases for the isolation of NA from a nucleic acid-containing starting material. According to the
30   invention the chaotropic guanidinium salt employed is preferably guanidinium thiocyanate (GuSCN).

The process according to the invention will usually be carried out in such a way that the starting material is mixed with sufficiently large amounts of chaotropic

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substance for instance guanidinium salt and for instance
silica particles to release essentially all of the nucleic
acid present in the starting material and bind it to said
silica particles. A suitable protocol is, e.g., the
5   addition of a suspension of silica particles to a buffered
GuSCN solution present in a reaction vessel, followed by
addition of the sample and thoroughly mixing. Then there
will take place lysis of cells and optionally present
viruses, and releasing NA will be bound to the silica
10 particles almost instantaneously. The resulting
silica-nucleic acid complexes will then be separated from
the liquid, e.g., by rapid sedimentation (centrifugation)
and disposal of the supernatant (e.g., by suction), and
then the complexes (e.g., in the form of a silica-nucleic
15 acid pellet) will be washed (redispersing or
homogenization), e.g., with a chaotropic guanidinium
salt-containing washing buffer using, e.g., a vortex mixer,
and sedimented again. Preferably, the silica-nucleic acid
complexes washed with washing buffer are further washed
20 successively with an alcohol-water solution (most
preferably about 70% ethanol to restrict losses in yield)
and with acetone, followed by drying to remove the acetone
(e.g., while heating). Then the NA present in the washed
and dried silica-nucleic acid complexes is eluted by means
25 of an aqueous elution buffer. The selection of the elution
buffer is co-determined by the contemplated use of the
isolated NA. Examples of suitable elution buffers are TE
buffer, aqua bidest and PCR buffer (see the part "Materials
and Methods"). Preferably, all of these steps are carried
30 out in a single reaction vessel (e.g., a 1.5 ml Eppendorff
tube of polypropylene), and the purified NA is recovered in
a relatively small volume, e.g., less than 100 μl. The
thus isolated NA is free from nucleic acid-degrading
enzymes and has such a high purity that it can immediately

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serve as a substrate for different enzymes, such as DNA polymerases (e.g., _Taq_-DNA polymerase), DNA restriction enzymes, DNA ligase, and reverse transcriptase (such as AMV reverse transcriptase).

5   In the process according to the invention, e.g., a sufficient amount of NA can be isolated from 50 µl whole blood, without preceding separation of plasma and cells, in about 45 minutes so as to demonstrate NA sequences by means of an amplification method such as the PCR method or the
10   so-called NASBA method as described in EP 0329822 (NASBA = nucleic acid sequence based amplification). The invention however, is also applicable to various other biological materials containing NA, such as serum, feces, urine, etc. For this reason the invention is useful in the diagnostics
15   of bacterial and viral infections, as well as in a study of gene polymorphisms within the scope of the prenatal diagnostics and the diagnostics of predisposition to hereditary tumours.

      In the method of NA isolation according to the
20   invention the risk of contamination is very low, because the whole procedure can be carried out in a single reaction vessel and the NA released from the crude starting material in the first step of the process is at least largely bound to the solid phase during the whole further purification
25   procedure. The risks for personnel, inherent to the processing of material possibly infected with viruses or bacteria, remain limited essentially to the first step of the isolation procedure in which the sample is placed in the reaction vessel. In this first treatment the
30   potentially present pathogens are efficiently inactivated. The process requires no special peripheral equipment (a vortex mixer, a centrifuge of the 12.000 g Eppendorff type and a waterbath or Eppendorff heating block belong to the standard laboratory equipment) and no specialist

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biochemical knowledge, so that the process is very suitable for routine NA isolation from large numbers of samples in other words for automation. By the process according to the invention more than 10 and even 24 or more different
5   samples can be processed in about 1 hour.

The invention not only relates to a process but also to a combination of means for isolating nucleic acid from a nucleic acid-containing starting material and a testkit in order to amplify the nucleic acid obtained by said
10   process.

In an embodiment a combination of means according to the invention comprises (a) a guanidinium (iso)thiocyanate-containing lysis buffer, (b) an aqueous suspension of silica particles having a particle size
15   ranging substantially between 0.05 and 500 μm, preferably between 0.1 and 200 μm and most preferably between 1 and 200 μm, (c) a guanidinium (iso)thiocyanate-containing washing buffer, and if required (d) an elution buffer.

Thus a combination of means according to the invention
20   may be composed of, e.g., the following 4 components:
component 1: a buffered guanidinium (iso)thiocyanate
            solution;
component 2: a suspension of silica particles;
component 3: a washing buffer: and (optionally)
25   component 4: an elution buffer.

If required, components 1 and 2 could be combined, which, however, leads to a limited shelf life.

Other reagents that are preferably used in the method of NA isolation according to the invention, such as ethanol
30   and acetone, belong to the standard laboratory equipment.

The invention will now be illustrated by a number of examples. In the preceding part the employed materials and methods will be described.

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MATERIALS AND METHODS

A) Suspension of Silica Coarse (SC)

Use was made of silicon dioxide (SiO_2), supplied by Sigma, having a particle size distribution of 0.5-10 μm,
5   80% of which ranged between 1 and 5 μm.

60 g silica were suspended in aqua bidest (up to a volume of 500 ml) in a cylinder having a diameter of 5 cm; the height of the aqueous column was 27.5 cm. After 1x g sedimentation for 25 hrs at room temperature supernatant
10   was sucked off, until 70 ml were left. Aqua bidest was added up to 500 ml, and the particles were resuspended by shaking the cylinder. After 1x g sedimentation for 5 hrs supernatant was sucked off, until 60 ml were left. After addition of 600 μl 32% (w/v) HCl the particles were
15   resuspended by vortexing. The suspension was made up in aliquots of 4 ml in 6 ml bottles, which were tightly closed and heated in an autoclave at 121°C for 20 min. This sedimentation protocol led to an enrichment of the larger silica particles, i.e. the particles having a particle size
20   above 1 μm, as was established by an electron-microscopic examination. Moreover, the autoclave treatment of an acid (pH about 2) silica suspension results in that optionally present nucleic acid is fully degraded. The thus obtained suspension of Silica Coarse will hereinbelow be referred to
25   as SC.

Suspensions of Silica derivatives

Silica   was   derivatized   with   methylacrylamide silicondioxide having alkyl-tails with a length of 2 to 18
30   C-atoms. The size of the derivatized silica particles varied from 63 to 200 μM. The pore size of the particles used was 500 Å. These silica derivates (12 $MAAMC_2$-C_{18}) were supplied by Diosynth, Oss.

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For the NA isolation (example H1) 0.5 g of the derivatized silica particles were suspended in 1 ml aqua bidest. These silica suspensions were pretreated with 120 µl 32% (w/v) HCl for 30 min. at 90°C.

5

Suspensions of Polystyrene latex particles

Use was made of two types of polystyrene latex particles. The polystyrene latex VQ69 red had been absorbed with sodium-dodecylsuccinate sulfate groups and has a particle size of 424 nm. The polystyrene latex VQ58B had a smaller size (328 nm) and no sulfate group has been absorbed on the outside.

Use was made of three, hydrophilic, glycidyl-methacrylaat polystyrene latex particles. The size of AGF27G; ACN3 red and AGY1515 were 933 nm, 206 nm and 846 nm respectively. All the mentioned polystyrene particles were supplied by ARLA-Arnhem.

Commercial filters

Use was made of

1. PVDF an Immobilon Transfer Membrane (hydrophobic) supplied by Millipore.

2. Nitrocellulose supplied by Schleicher and Schuell (0,2 µM Ref.no.401.396).

3. Hybond-N a Nylon Hybridization membrane (0,45 micron, lot: 16872) supplied by Amersham.

B) L2 buffer

L2 buffer (0,1 M Tris.Cl pH 6.4) was prepared by dissolving 12.1 g TRIS (Boehringer) in 800 ml aqua bidest, adding 8.1 ml 37% (w/v) HCl and bringing the volume to 1 litre with aqua bidest.

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C) <u>Washing liquid L2</u>

The washing liquid L2 was prepared by dissolving 120 g GuSCN (guanidine thiocyanate of Fluka) in 100 ml L2 buffer.

5   <u>Washing liquids L2*</u>

The washing liquid L2* was prepared by dissolving 12.45 g KI (potassium iodide from Merck) in 25 ml L2-buffer.

For preparing a NaI based chaotropic substance, 11.25 10   g NaI (sodium iodide from Merck) was dissolved in 25 ml L2-buffer. For a sodium thiocyanate based chaotropic substance, 6.1 g NaSCN (Baker) was dissolved in 25 ml L2-buffer.

For preparing a chaotropic substance containing KI and 15   urea (8M) 12.45 g KI and 12.0 g urea were dissolved in L2-buffer (25 ml). Similarly chaotropic substances combining urea with NaI and urea with NaSCN were prepared.

D) <u>Lysis buffer L5</u>

20   The lysis buffer L5 was prepared from 100 ml L2 buffer by dissolving therein 120 g GuSCN (gently shaking in a warm water bath of about 60°C), then adding 26.0 g of 40% (w/v) Dextran sulfate (Pharmacia LKB) solution, 22 ml of 0.2 M EDTA pH 8, and 2.6 g Triton X-100 (Packard), and then 25   homogenizing the solution. The 0.2 M EDTA pH 8 solution was prepared by dissolving 37.2 EDTA (Titriplex of Merck) and 4.4 g NaOH (Merck) in 500 ml water.

E) <u>Lysis buffer L6</u>

30   The lysis buffer L6 was prepared from 100 ml L2 buffer by dissolving therein 120 g GuSCN (gently shaking in a water bath of 60°C), then adding 22 ml of 0.2 M EDTA pH 8, and 2.6 g Triton X-100 (Packard) and then homogenizing the solution.

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Lysis buffer L6*

The lysis buffer L6* was prepared from 25 ml L2-buffer
by dissolving therein 12.45 g KI (potassium iodide, Merck)
(gently shaking in waterbath of 40°C) subsequently adding
5   5.5 ml of 0.2 M EDTA (pH 8.0) and 0.65 g Triton X-100
(Boehringer 789704) and finally homogenizing the solution.
The same procedure was applied for lysisbuffer L6* with NaI
(sodium iodide, Merck) and lysisbuffer L6* with NaSCN
(sodium thiocyanate, Baker).

10 The lysisbuffer L6* with combination KI and urea was
prepared from 25 ml L2-buffer by dissolving therein 12.45
g KI (potassium iodide, Merck) and 12.0 g urea (Gibco BRL).
Subsequently 5.5 ml of 0.2 M EDTA (pH 8.0) and 0.65 g
Triton X-100 (Boehringer) were added and the mixture was
15 homogenized.   The same   method   was   followed   for   the
preparation of NaI/urea and NaSCN/urea.

F) Lysis buffer GEDTA

By GEDTA is meant a solution of 120 g GuSCN in 100 ml
20 0.2 M EDTA pH 8.

G) TE buffer

A buffer suitable for elution is a 10 mM Tris.Cl, 1 mM
EDTA solution with pH 7.5 (TE buffer), if desired
25 comprising 0.5 U/μl RNAsin (Promega).

H) Test tubes

The test tubes were assembled on the same day as the
extraction procedure by adding 900 μl lysis buffer and 40
30 μl of an NA carrier (latex beads or silica, such as SC, or
diatomaceous earth) to Eppendorff centrifugal tubes (type
3810, 1.5 ml).

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I) <u>Washing procedure</u>

A pellet is washed by adding 1 ml washing liquid, then vortexing until the pellet is resuspended, centrifuging for 15 sec. at 12000x g, and discarding the supernatant by
5   suction.

J) <u>Elution procedure</u>

The elution takes place by adding at least 25 μl, preferably at least 40 μl elution buffer, vortexing briefly
10   (2 sec) and incubating for 10 min. at 56°C.

K) <u>Protocol B</u>

This protocol is suitable for isolating dsDNA from complex starting materials, such as human serum, whole
15   blood, watery feces or urine and makes use of Eppendorff test tubes with 900 μl GEDTA and 40 μl SC.

1.   Vortex test tube until pellet is resuspended.

2.   Add 50 μl starting material (e.g., serum, whole blood, feces or urine) and vortex immediately until
20   homogeneous (5-10 sec.).

3.   Leave at room temperature for 10 min. and vortex 5 sec.

4.   Centrifuge for 15 sec. at 12000x g and discard supernatant by suction.

25   5.   Wash pellet once with GEDTA.

6.   Wash pellet twice with 70% ethanol.

7.   Wash pellet once with acetone.

8.   Dry pellet for 10 min. at 56°C with open lid.

9.   Elute DNA with 50 μl TE buffer without RNAsin.

30   10.   Centrifuge for 2 min. at 12000x g; supernatant contains DNA.

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L. Protocol Y

This protocol is suitable for isolating NA (simultaneous purification of dsDNA, ssDNA, dsRNA and ssRNA) from complex starting materials, such as human serum, whole blood, watery feces or urine and makes use of Eppendorff test tubes with 900 μl L6 and 40 μl SC.

1. Vortex test tube until pellet is resuspended.

2. Add 50 μl starting material (serum, whole blood, feces or urine) and vortex immediately until homogeneous (about 5 sec.).

3. Leave at room temperature for 10 min. and vortex 5 sec.

4. Centrifuge for 15 sec. at 12000x g and discard supernatant by suction.

5. Wash pellet twice with L2.

6. Wash pellet twice with 70% ethanol.

7. Wash pellet once with acetone.

8. Dry pellet for 10 min. at 56°C with open lid.

9. Elute NA with 50 μl TE buffer, optionally in the presence of RNAsin.

10. Centrifuge for 2 min. at 12000x g; supernatant contains NA.

Protocol Y*

This protocol is suitable for isolating NA from complex starting materials, such as human serum, urine or bacterial cultures.

Procedure:

Eppendorff tubes were used with 900 μl L6* and 40 μl SC.

1. Vortex test tube until pellet is resuspended.

2. Add 50 μl starting material (serum-plasmid, urine-plasmid mixtures or overnight bacterial culture) and vortex immediately until homogeneous (5 sec.).

3. Leave at room temperature for 10 min. while mixing.

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4.   Centrifuge for 15 sec. at 14.000 g discard supernatant
     by suction.
5.   Wash pellet twice with L2* washing liquid.
6.   Wash pellet twice with 70% ethanol.
7.   Wash pellet once with acetone.
8.   Dry pellet for 10 min. at 56°C with open lid.
9.   Elute NA with 50 µl TE-buffer (10 mM Tris-1mM EDTA pH
     8.0) optionally in the presence of RNAsin.
10. Centrifuge for 2 min at 14.000 g; supernatant contains
     NA.

     Protocol Y**

     This protocol is suitable for isolating NA in presence
of GuSCN as chaotropic substance and a filter capable of
binding NA (see Materials & Methods) . The NA detection was
performed by polymerase chain reaction by bringing this
filter directly in the polymerase chain reaction mix, thus
without prior elution of the NA from the filter.
Procedure:
Eppendorff tubes were used with 900 µl L6 lysis buffer and
a filter (size 1 cm/1 cm)
1.   Add 50 µl nucleic acid containing solution and vortex
     the test tube briefly.
2.   Leave at room temperature for 10 min. while mixing.
3.   Discard supernatant.
4.   Wash filter twice with L2 washing liquid.
5.   Wash filter twice with 70% ethanol.
6.   Dry filter 10 min. at 56°C with open lid.
7.   A small piece of the filter was directly added to the
     polymerase chain reaction solution.

M)   Protocol Z

     This protocol is suitable for isolating NA from
complex starting materials, such as human serum, whole
blood, watery feces or urine and makes use of Eppendorff
test tubes with 900 µl L5 and 40 µl SC. The isolated NA

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can be used for hybridization reactions but is less suitable as a substrate for restriction enzymes. T4 DNA ligase, however, is active. As compared with protocol Y, this protocol Z leads to higher NA yield.

5   1.   Vortex test tubes until pellet is resuspended.
     2.   Add 50 μl starting material (serum, whole blood, feces or urine) and vortex immediately until homogeneous (about 5 sec.).
     3.   Leave at room temperature for 10 min. and vortex 5
10   sec.
     4.   Centrifuge for 15 sec. at 12000x g and discard supernatant by suction.
     5.   Wash pellet twice with L2.
     6.   Wash pellet twice with 70% ethanol.
15   7.   Wash pellet once with acetone.
     8.   Dry pellet for 10 min. at 56°C with open lid.
     9.   Elute NA with 50 μl TE buffer, optionally in the presence of RNAsin.
     10.   Centrifuge for 2 min. at 12000x g; supernatant
20   contains NA.

N) Starting materials

       The examples are divided into sections as follows, inter alia (sections A-D) in accordance with the nature of
25 the starting material:
    section A: human serum
    section B: human whole blood
    section C: human urine
       These sections A, B and C are especially meant to show
30 that both dsDNA and ssRNA can be isolated in pure form.
    section D: human feces
       This section D shows, among others, that the dsRNA can also be isolated.
    section E: single stranded DNA

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This section E comprises experiments showing that the invention can be used for isolating ssDNA.
section F: diatomaceous earth

This section F shows that diatom skeletons are very
5   useful as the silica particles to be used according to the invention. It is also shown that the invention can be used for isolating NA from different gram-negative bacteria. Section G shows that NA can be purified from bacterial cells using various chaotropic substances.

10   Section H and I show the isolation of DNA with the aid of alternative solid phases.

There was always used an amount of 50 µl. The blood used in section B and F was always fresh blood drawn off in the presence of EDTA to prevent clotting (using the
15   Venoject system of Terumo N.V., Louvain, Belgium, collecting tubes of the type VT-574 TKZ). The starting materials used in the other sections (serum, urine and feces) were frozen. In examples A1, A2, A3, B1, B2, B5, B7 and F1 the serum or blood was from the same subject.
20

O) Further methods

For gel-electrophoretic examination, part of the eluted amount of NA was loaded on a neutral agarose-gel containing 1 µg/ml ethidium bromide in the buffer system
25   described by Aaij and Borst (Biochim.Biophys.Acta 269, 1972, 192). Photographs were taken under UV illumination of the gel.

In some experiments a known amount of a purified DNA (input DNA) was added to the clinical sample. In these
30   cases an amount of input DNA corresponding to an extraction efficiency of 100% was also loaded on the same gel.

Bacterial plasmid DNA was purified as described by Ish-Horowicz and Burke (Nucleic Acids Res. 9, 1981, 2989) from Escherichia Coli HB101, followed by column

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chromatography with Sepharose CL 2B (Pharmacia, Inc.) and ethanol precipitation. Bacterial plasmid DNA was purified from Escherichia Coli JM101 (J. Messing, Rec. DNA Techn. Bull. 2:43-48(1979) as described by Birnboim and Doly (Maniatis, T. et al., Molecular Cloning, CSH, New York). The pCMV-E contains a 0.4 kb human cytomegalo virus DNA fragment cloned in the 2 kb vector pHC 624 (Boros in Gene 30, 1984, 257); pEBV-10 contains a 0.9 kb Epstein Barr virus DNA fragment cloned in the same vector. To obtain a plasmid preparation enriched for relaxed circular (CII) molecules, pEBV-10 DNA (2.9 kb) was treated with DNAse I. Component II molecules serve as a model for purification of Hepatitis B viral DNA which is present in virions as a 3.2 kb relaxed circular DNA molecule.

The pGem3p24 contains a 1,45 kb HIV sequence; the construction of pGem3p24 is described below.

The sequence of HIV HxB2 DNA has been described by several authors (J. Virol. 61, 633-637(1987); Nature 326, 711-713(1987); Aids Res. Hum. Retrovirus 3, 41-55(1987); Aids Res. Hum. Retrovirus 3, 33-39(1987) and Science 237, 888-893(1987)).

HIV HxB2 DNA was partially cleaved with FokI at sites 1189 and 2613 of the original HIV HxB2 sequence. The nucleotide nrs. refer to the Genebank designation.

The FokI sites of this fragment were filled up using Klenow DNA polymerase (Maniatis, vide supra) and cloned (Maniatis, vide supra) in the polylinker SmaI-site of plasmid pUC-19. The resulting plasmid which carries the HIV HxB2 DNA fragment was called pUC19-p24.

To obtain plasmid pGem3p24, the 1450 bp EcoRI-BamHI fragment of pUC19-p24 was cloned in the EcoRI-BamHI digested vector pGem3 (2867 bp; Promega Corporation, Madison USA).

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The primers used in the PCR method were synthesized on
an oligo-synthesizer apparatus (from Applied Biosystem).
Nucleotide sequence of the primers ES47 (25 mer) and ES75
(47 mer) are given below.

5   ES47

              10   20
      ACAGGAGCAG ATGATACAGT ATTAG

10   ES75

              10   20   30   40
      AATTCTAATA CGACTCACTA TAGGGCCTGG CTTTAATTTT ACTGGTA

15

In most of the RNA isolation experiments (examples A3,
B5, B6, B7, C2, D1, E1, F1 and F2) no precautions were
taken other than the optional use of RNAsin in the elution
20   buffer to avoid RNA degradation during the purification
procedure.   Gloves were only worn during the addition of
the clinical samples to the test tubes; no RNAse inhibitors
were used for the preparation of the reagents; and
non-autoclaved Eppendorff vessels and pipette tips were
25   used.   Examples F1 and F2, among others, have shown that
the presence of RNAsin during elution is not strictly
necessary.
The enzymes used were commercially available and were
used as recommended by the manufacturer.   All restriction
30   enzymes, as well as RNAse A, T4 ligase and AMV reverse
transcriptase were from Boehringer (Mannheim).   Taq-DNA
polymerase was from Cetus Inc.   The polymerase chain
reactions (PCR) were performed with a Perkin Elmer Cetus
DNA-thermal cycler.
35   For different uses it is of essential importance that
the reagents used in the process according to the
invention, especially the NA carrier (for instance silica

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particles) and the lysis and washing buffers containing the chaotropic substance, should not be impurified by nucleic acid (e.g., by NA containing bacteria or viruses). This can be ensured for the NA carrier by heating it in an autoclave for 20 min. at 121°C. However, this method is not useful in the GuSCN-containing lysis and washing buffers (GEDTA, L5, L6, and L2), both by reason of a possible loss of activity and because of the attendant risk for the environment. In order to make these reagents (as much as possible) nucleic acid-free, they may be passed through a column of silica particles in accordance with the invention. Due to the lysing properties of the GuSCN-containing buffers and the property of silica to bind NA in the presence of the chaotropic substance GuSCN, such a procedure leads to an NA-free buffer. The column itself can be made nucleic acid-free by heating for, e.g., one ore more hours at, e.g., 500°C or more.

P) <u>DNA types</u>

CI	:	Covalently closed circular DNA (plasmid)
CII	:	relaxed (nicked) circular DNA (plasmid)
CIII	:	Linear DNA (linearized plasmid)
LMW	:	low molecular weight DNA (< 0.5 kb); HpaII digest of pHC 624, fragments of 471 bp, 404 bp, 242 bp (2 fragments), 190 bp, 147 bp, 110 bp, 67 bp and some smaller fragments of undetermined lengths.
MMW	:	medium molecular weight DNA (0.5-29 kb); HindIII digest of phage lambda DNA, fragments of 23 kb, 9.4 kb, 6.7 kb, 4.4 kb, 2.3 kb, 2.0 kb and 0.56 kb.
HMW	:	high molecular weight DNA (> 29 kb).
ssDNA	:	phage M13mp9 single stranded DNA (Boehringer).

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SECTION A: DNA/RNA purification from human serum

In human serum NA can be present, e.g. in viruses or bacteria. These organisms can occur both in free form and also bound in immune complexes. The amounts of NA are usually so low that detection through agarose gel electrophoresis and UV illumination of ethidium bromide/NA complexes is impossible. To show that DNA can be purified from human serum, microgram quantities of purified DNA were added to the serum, and then the DNA was isolated according to protocol B (examples A1 and A2). To show that DNA and RNA can be simultaneously purified from human serum, cultured mammalian cells or bacteria (carrying a small plasmid) were added to the serum, and then NA was isolated according to protocol Y (Example A3). Finally, example 4 shows that, by protocol Y, RNA present in human serum can be purified from HIV (Human Immunodeficiency Virus) and can be detected by the PCR method. Example A5 shows that, by protocol Y*, DNA in human serum can be purified using various chaotropic substances in combination with silica as nucleic acid binding solid phase.

Example A1: DNA purification from human serum

Human serum (500 μl) was mixed with known amounts of purified DNA [100 μl LMW (45 μg), 20 μl MMW (20 μg), 40 μl CI/II (20 μg)] and 10 samples of 66 μl were used as input material for 10 DNA extractions according to protocol B. The amount of SC (suspension of Silica Coarse) present in the test tubes was varied in this experiment between 2.5 and 40 μl. The extractions were carried out in duplicate and half (30 μl) of the eluted DNA from each sample was electrophoresed through a 1% agarose gel. For comparison, half of the amount of input DNAs were also loaded on the same gel in control lanes.

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Double-stranded DNA, both linear (range 23 kb to approximately 60 bp), covalently closed (CI) and relaxed circular (CII) DNA were efficiently isolated if the amount of SC exceed 10 μl. The yield of the largest MMW fragment

5   (approx. 23 kb) seems relatively low when compared to the smaller fragments, which in view of other experiments, may be attributed to shearing of high molecular weight fragments.

The control lanes show respectively the amount of LMW,

10   CII/CI and MMW DNA which would be found in an extraction efficiency of 100%. As previously stated, a CII-rich (DNAse I-treated) 3 kb plasmid (pEBV-10) was used as input material.

15   Example A2: DNA isolated from human serum is a good
                  substrate for restriction enzymes and T4 DNA
                  ligase

Purified DNA preparations were added to 12 human serum samples of 50 μl. DNA was isolated from these 12 mixtures

20   according to protocol B; elution was effected with 50 μl TE. Half of the eluted DNA was treated (in duplicate) either with one of the following three restriction enzymes: EcoRI, BamHI and BglII (these are active in low-salt, medium-salt and high-salt buffers, respectively), or

25   treated with T4 DNA ligase, or not treated. The DNA samples were electrophoresed through a 1% agarose gel and visualized by UV illumination.

The results of the T4 ligase treatment (1 h at 37°C, 3 units of T4 ligase in a 30 μl reaction volume) shows a

30   shift in molecular weight of the DNA fragments and indicates that the DNA isolated from human serum is not significantly affected by exonucleolytic degradation.

The results for 8 serum samples to which a purified plasmid (pCMV-E; 3.3 μg; 1.5 μl) was added shows

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respectively that for EcoRI, BamHI and BglII digests all
restriction   enzymes   linearized   the   plasmid.   All
restriction enzyme incubations were done in a 30 μl
reaction volume for 1 h at 37°C with 9 units of enzyme.

5

Example A3: simultaneous isolation of DNA and ssRNA
            from a human serum

         Since in human serum only very low levels of RNA are
present (e.g., in viruses, bacteria or cells) which are not
10   detectable by UV illumination of ethidium-bromide stained
gels, exogenous RNA sources were added to human serum
samples.   Mammalian cells or bacteria were used as
exogeneous RNA sources.   The NA was isolated from the
samples according to protocol Y and eluted in 50 μl TE with
15   0.5 U RNAsin per μl in the absence or in the presence of
RNAseA (40 ng per μl of the elution buffer).   The results
of the subsequent electrophoresis through a 1% agarose gel
shows that RNA and DNA can be detected.   The mammalian
cells added were per 50 μl serum sample 5×10^5 rat 10B cells
20   (Boom et al., J. Gen. Virol. 69, 1988, 1179) while the
bacteria added were per 50 μl serum the cell pellet of a
100 μl overnight culture of the E. coli strain HB101
containing the plasmid pCMV-E.

25   Example A4: Polymerase chain reaction for the detection
            of Human Immunodeficiency Virus RNA isolated
            from human serum

         NA (75 μl) was isolated from 2 human serum samples of
50 μl each (patients F and H) according to protocol Y. The
30   serum of patient F contained a high (2700 pg/ml) level of
the HIV antigen P24 (according to the HIV P24 antigen solid
phase immunoassay of Abbott Laboratories) but was negative
for HIV antibodies (according to the HIV antibodies ELISA

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of Abbott Laboratories), and the serum of patient H was
negative in both tests.

5

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Part of the isolated NA (43 μl) was treated with
RNAse-free DNAse (Boehringer; 1 U DNAse/μl) for 90 min at
37°C.   After ethanol precipitation and heat inactivation
for 15 min at 68°C, the RNA was suspended in 15 μl TE
buffer. A 5 μl portion of this RNA preparation was either
or not treated with 0.4 U/μl AMV reverse transcriptase (30
min at 42°C; reaction volume 20 μl) in the presence of HIV
specific primers.   Then the reaction volume was made up to
100 μl by adding 80 μl of 1.25 x concentrated PCR buffer
including dNTPs, 1 U of Taq-DNA polymerase was added, and
the amplification was started (1 cycle comprised 1 min at
95°C, 1 min at 55°C, 2 min at 72°C).   10 μl aliquots were
taken from the reaction mixtures at 20, 25, 30 and 35
cycles and were applied to a 2% agarose gel. The expected
330 bp HIV amplimer fragment was already observed after 25
cycles for patient F RNA that had been treated with reverse
transcriptase, suggesting that HIV RNA was present in his
serum.

Example A5: DNA purification with several chaotropic
            substances

25

30

Ten 50μl samples of human serum were mixed with each
10 μg of purified pGem3p24 DNA consisting of CI and CII
form (see methods).   These 10 plasmid/serum mixtures were
used as input material for extractions according to
protocol Y*.   For the concentrations of chaotropic
substances used see Table A5.1.

After extraction 25% of the eluted DNA from each
sample was analyzed on 0.8% agarose gel.   To allow
quantitation of plasmid DNA recovery input DNA was also
loaded directly on the same gel.

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After electrophoresis the gels were photographed under
UV illumination and the efficiency of DNA recovery was
visually judged on the basis of the plasmid bands
intensities (see legend of table A5.1).

Similarly experiments were performed using NaI and
NaSCN as chaotropic substances (see sample description
below).

Table A5.1

Efficiency of recovery of plasmid DNA from human serum
samples using various chaotropic substances in combination
with silica.

Sample nr.	chaotropic substance used	Recovery of pGEM3p24:CII	Recovery of pGem3p24:CI
1	GUSCN	++	±
2	KI 3M	-	-
3	KI 3M/urea 1M	-	-
4	KI 3M/ureum 8M	++	+
5	NaI 3M	-	-
6	NaI 3M/urea 1M	-	-
7	NaI 3M/urea 8M	++	+
8	NaSCN 3M	-	-
9	NaSCN 3M/urea 1M	±	±
10	NaSCN 3M/ urea 8M	++	+

Legend:

10 detectable samples prepared as described above and using
the chaotropic substances indicated in the table were
analyzed.

-: no recovery   ±: little recovery

+: visible recovery   ++: quantative recovery

The results summarized in Table A5.1 show that
covalently closed (CI) and relaxed circular (CII) pGem3p24

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DNA were efficiently isolated when 3M KI, 3M NaI or 3M NaSCN in combination with 8M urea were used as chaotropic substances. The yield of CII seems relatively high when compared with CI.

SECTION B: DNA/RNA purification from human whole blood

One ml of human blood contains approx. 5×10^9 erythrocytes which are non-nucleated and do therefore not contribute to the NA amount of blood. The NA amount of blood is largely determined by the white blood cells (approx. $4-10 \times 10^6$ per ml). These cells are embedded in an aqueous medium (plasma) containing large amounts of proteins (approx. 70 mg/ml blood). Thus, whole blood is an extremely unpure source for NA purification. The examples of section B show that notwithstanding NA can be isolated from whole blood by protocols B and Y.

Example B1: DNA isolation from human whole blood

Human blood (500 μl) was mixed with known amounts of purified DNA 100μl LMW (45 μg), 80 μl CI/II (40 μg) and 10 samples of 68 μl were used as input material for 10 DNA extractions according to protocol B. In this experiment the amount of SC (suspension of Silica Coarse) present in the test tubes was varied between 2.5 and 40 μl. The extractions were carried out in duplicate and half (30 μl) of the eluted DNA from each sample was electrophoresed through a 1% agarose gel. For comparison, half of the amount of input DNAs was also loaded on the same gel.

Double-stranded DNA, both linear, covalently closed (CI) and relaxed circular (CII) DNA, was efficiently isolated from human whole blood if more than 10 μl SC, were used. The amount of DNA recovered from whole blood was proportional to the amount of SC up to approximately 10 μl. Higher amounts seemed to be saturating.

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Example B2: DNA isolated from human whole blood is a
             good substrate for restriction enzymes and T4
             DNA ligase

Purified DNA preparations were added to 12 human blood
5   samples of 50 μl. The DNA was isolated from these 12
     mixtures according to protocol B; elution occurred with 50
     μl TE. Half of the eluted DNA was either treated with one
     of the following three restriction enzymes: EcoRI, BamHI
     and BglII (these are active in low-salt, medium-salt and
10   high-salt buffers, respectively), or treated with T4 DNA
     ligase, or not treated. The DNA samples were
     electrophoresed through a 1% agarose gel and visualized by
     UV illumination.

          The results of T4 ligase treatment (1 h at 37°C, 3
15   units of T4 ligase in a 30 μl reaction volume) shows a
     shift to a higher molecular weight of the DNA fragments and
     indicates that the DNA isolated from human blood is not
     significantly affected by exonucleolytic degradation.

          The results for 8 blood samples to which a purified
20   plasmid (pCMV-E; 3.3 μg; 1.5 μl) was added show that for
     EcoRI, BamHI and BglII digests all restriction enzymes
     linearized the plasmid. All restriction enzyme incubations
     were done in a 30 μl reaction volume for 1 h at 37°C with
     9 units of enzyme.

25

Example B3: DNA isolation from 10 different samples of
             blood

          In this example 10 different samples of human blood
     randomly chosen from a blood bank are used as starting
30   material. Of each of the samples the number of white blood
     cells (WBC) was known. DNA was purified from 50 μl of the
     samples according to protocol B, and elution occurred with
     75 μl TE. One third of the isolated DNA was directly

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applied to a 1% agarose gel and part (2 μl) of the remainder was used for a PCR.

The same samples were subjected to the same isolation procedure after 3 μl LMW-DNA (6 μg) was added to each 50 μl sample. Here, too, 25 μl of the eluate (75 μl) was directly applied to the gel; another portion of 25 μl of the eluate was first treated with T4 DNA ligase (1 h at 37°C, 2 U in a reaction volume of 30 μl) and then applied to the same gel.

The content of white blood cells (WBC) of blood samples 1-10 was as follows:

sample No.	WBCx10⁹/1	sample No.	WBCx10⁹/1
1	4.9	6	8.3
2	5.1	7	8.5
3	5.9	8	9.2
4	6.7	9	10.3
5	7.7	10	10.5

Example B4: polymerase chain reaction for the detection of the human beta-globin gene in human white blood cells

To show that DNA isolated from human whole blood according to protocol B is a good substrate for Taq-DNA polymerase, 2 μl of the DNA isolated from ten different blood samples according to example B3 was subjected to a PCR with beta-globin specific primers. The PCR comprised 32 cycles, each cycle being 1 min at 94°C and then 3 min at 65°C. Part of the amplimers (50%) was electrophoresed through a 2% agarose gel. A 120 bp amplimer and the primer bands could be detected.

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Example B5: simultaneous purification of DNA and ssRNA
         from human blood (reproducibility)

        To show that DNA and RNA can be purified from human
blood in a reproducible manner, 6 blood samples of each 50
5   µl from one person were subjected to protocol Y, the NA
being eluted in 75 µl TE with RNAsin (0.5 U/µl). A 25 µl
portion of the eluate was applied to a neutral 1% agarose
gel and electrophoresed. The results show that DNA and RNA
can be detected.
10

Example B6: simultaneous purification of DNA and ssRNA
         from human blood (10 different samples)

        Blood samples of 50 µl from 10 different persons (see
example B3) were subjected to protocol Y, the NA being
15   eluted with 40 µl TE with 0.5 U/µl RNAsin. Eluate portions
of 30 µl were electrophoresed through a neutral 1% agarose
gel. The result shows that both DNA and RNA can be
detected.

20   Example B7: simultaneous purification of DNA and ssRNA
         from human blood

        Exogenous RNA sources were added to samples of a human
blood. Mammalian cells or bacteria were used as exogenous
RNA sources. The NA was isolated from the samples
25   according to protocol Y and eluted in 50 µl TE + 0.5 U/µl
RNAsin in the absence or in the presence of RNAseA (40 ng
·per µl of the elution buffer). Per 50 µl blood sample
$5x10^5$ rat 10B cells (Boom et al., J.Gen.Virol. 69, 1988,
1179) were added as mammalian cells, and per 50 µl blood
30   the cell pellet of a 100 µl overnight culture of the E.coli
strain HB101 containing the plasmid pCMV-E was added as
bacteria.

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The results show that both mammalian ssRNA (18S and 28S ribosomal RNAs) and bacterial ssRNA (16S and 23S ribosomal RNAs) can be purified from human whole blood.

In addition, genomic DNA and plasmid (form I) DNA are
5   efficiently recovered.

SECTION C: DNA/RNA purification from human urine

In human urine, NA can be present, e.g., in viruses or bacteria and in cells from the urinary tract. The amounts
10   are usually so low that detection through agarose gel electrophoresis and UV illumination of ethidium bromide/NA complexes is impossible. To show that DNA can be purified from human urine, microgram quantities of purified DNA were added to urine, and the DNA was subsequently isolated
15   according to protocol B (example C1). To show that DNA and RNA can be simultaneously purified from human urine, cultured bacteria (carrying a small plasmid) were added to urine, and the NA was subsequently isolated according to protocol Y (example C2).

20   Example C3 shows that DNA can be purified from human urine with alternative chaotropic substances such as KI, NaI and NaSCN instead of GuSCN with silica as nucleic acid binding solid phase according to protocol Y*.

25   Example C1: DNA purification from human urine

3 μl LMW DNA (6 μg) was added to 10 randomly chosen human urine samples of 50 μl with varying turbidity (samples 4, 5, 6 and 7 were clear, samples 1, 2, 3 and 8 were slightly turbid, and samples 9 and 10 were very
30   turbid). The DNA was isolated according to protocol B and eluted with 75 μl TE buffer. One third of each eluate was applied to a 1% agarose gel. Another part of 25 μl was treated with a 1.8 U T4 DNA ligase (1 h at 37°C in a 30 μl reaction volume) and applied to the same gel. Marker lanes

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contain respectively LMW DNA and MMW DNA. The amount of
LMW DNA (2 µg) in a marker lane represents the amount to be
observed with an extraction efficiency of 100%.

5   The results show that DNA can be efficiently purified
from human urine with protocol B and is a good substrate
for T4 DNA ligase.

        The LMW DNA isolated from urine sample No. 10 has been
clearly degraded. It was to be expected, however, that
naked DNA (as used in this experiment) would be degraded if
10   a urine sample is rich in nucleases. Degradation is
therefore likely to have taken place previously during the
preparation of the urine/DNA mixtures rather than during
purification. The next example (C2) shows that DNA and
even ssRNA present in cells (as opposed to naked) can be
15   efficiently recovered from urine sample No. 10.

Example C2: simultaneous purification of DNA and ssRNA
            from human urine

        In this experiment the same 10 urine samples as used
20   in example C1 were mixed with bacteria carrying a 2.4 kb
plasmid (pCMV-E). The NA was isolated from these mixtures
according to protocol Y and eluted in 75 µl TE buffer with
0.5 U/µl RNAsin. One third of the eluate was
electrophoresed through a 1% agarose gel. Another 25 µl
25   portion of the eluate was treated with 10 U of the
restriction enzyme EcoRI which linearizes pCMV-E (1 h at
37°C in a 30 µl reaction volume). This treatment was
conducted in the presence of 40 ng/µl RNAseA. The
electrophoresis result shows the 23S and 16S ribosomal
30   RNAas as well as the covalently closed (CI) and linear
(CIII) forms of plasmid DNA.

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Example C3: DNA purification with other chaotropic
                    substances

    Human urine (50 µl) was mixed with 400 µl chaotropic
substance, lysis buffer L6* and 1 µg pGem3p24 DNA. This
5   total suspension was mixed and added to 500 µl chaotropic
substance (see table C3.1) and 40 µl SiO_2 for the
purification of DNA according to protocol Y*. The quantity
of DNA isolated from urine was analysed using agarose gel
electrophoresis. Efficiency of DNA recovery was judged as
10   described in Example A5 and the results are summarized in
Table C3.1.

Table C3.1 Recovery of plasmid DNA from human urine
                    samples using various chaotropic substances
15   in combination with Silica (see also legends
                    of Table A5.1)

Sample nr	chaotropic substance used	recovery of pGem3p24 CII	Recovery of pGem3p24 CI
1	$GuSCN/SiO_2$	+	+
2	Ki 3M/SiO_2	+	+
3	NaI 3M/SiO_2	+	+
4	NaSCN 3M/SiO_2	+	+

    Table C3.1 shows that the yields for DNA bands CI-type and
25   CII-type plasmid DNA were the same.

Example D1: purification of rotaviral dsRNA from human
                    feces

    Members of the virus family Reovirdae possess a genome
30   consisting of double stranded RNA. Important pathogens
belonging to this family are the Rotaviruses which can
cause serious diarrhoeas and are then present in vast

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amounts in feces samples. The rotaviral genome consists of 11 dsRNA segments (see Hishino in J. Clin. Microbiol. 21, 1985, 425) which could be isolated from feces supernatant according the protocol B. 100 μl supernatant obtained by 2
5   min. centrifugation of the diarrhoea sample at 12000xg were used for the isolation.

The results using samples from 6 different patients with proven rotaviral infection (proven by the Wellcome Rotavirus latex test and by the Kallestad Pathfinder
10   Rotavirus direct antigen detection system) prove that dsRNA can be extracted.

Similar results (usually with higher rotaviral dsRNA yields) were obtained when the first centrifugation step was omitted and the feces samples were directly used as
15   input material for protocol B or Y.

Example E1: purification ssDNA from human blood, serum
          and urine

To show that single stranded DNA can also be isolated
20   from clinical samples, 1 μg (4 μl) of purified phage M13 DNA (M13mp9 DNA, Boehringer) was added to 50 μl human serum, human blood or human urine and purified according to protocol B or according to protocol Y. All the extractions were carried out in quadruplicate. DNA was eluted in 50 μl
25   TE buffer, and 25 μl were electrophoresed through a 1% agarose gel. A marker lane contains 500 ng of M13 ssDNA.

The results show that single stranded DNA can be isolated from human blood, serum or urine by protocol Y
30   and, to a lesser extent, by protocol B.

SECTION F: binding of NA to diatomaceous earth

Since the skeletons of diatomaceous earths consist almost completely of SiO_2, it was examined whether they

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might serve as the silica to be used. Of each of five
different commercially available diatomaceous products
[Celatom FW14, Celatom FW50, Celatom FW60, Celite (AK) and
Celite 521, Janssen Biochimica, Louvain, Belgium] 10 g were
5   mixed with 50 ml aqua bidest and 500 μl 37% HCl, followed
by heating the resulting suspensions in an autoclave to
121°C for 20 min. In examples F1 and F2 the thus obtained
suspensions were used for NA extractions according to
protocol Y.
10

Example F1: NA isolation from human blood

    Human blood was mixed with E.coli HB101 bacteria,
carrying the plasmid pCMV-E, and the bacterial pellet of
100 μl of an overnight culture were added to 50 μl blood.
15   Samples of 50 μl were used as input material for NA
extractions according to protocol Y. Instead of 40 μl SC,
40 μl of the above suspensions of diatomaceous earth were
used. The NA was eluted in 75 μl TE buffer, without using
RNAse inhibitor, and 20 μl of the eluate were directly
20   applied to the gel. Another portion of 20 μl of the eluate
was treated with RNAse A (40 ng/μl) together with 9 U BamHI
for 1 h at 37°C in a reaction volume of 25 μl and then
applied to the gel.
    A marker lane contains 1 μg MMW DNA.
25   The results show that the diatomaceous earth
suspensions have NA binding properties similar to SC. Both
dsDNA (component I molecules) and ssRNA (23S and 16S rRNAs)
were bound.   Plasmid DNA was sufficiently pure to be
completely linearized (component III) by BamHI.
30

Example F2: NA purification from gram-negative bacteria

    9 Different species of gram negative bacteria known to
cause disease in humans were cultured on solid agar plates.
Of each of these bacterial species 5 to 10 μl was scraped

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off the plates and used as input material for NA extractions according to protocol Y, and 40 μl SC or 40 μl of the Celite 521 suspension were used as NA carrier.

    The extractions in which SC was used had to be stopped
5 during the first wash since the NA silica complexes could no longer be homogenized, not even after vortexing for a long time (over 3 min.). On the other hand, extractions in which Celite 521 was used proceeded without problems, presumably due to the larger particle sizes of the
10 diatomaceous earth relative to the SC particles. The NA was eluted with 70 μl TE buffer without RNAsin and part of the eluate (20 μl) was electrophoresed through a 1% agarose gel.

    The marker lanes contain 1 μg MMW DNA. Results for
15 the following types of bacteria were obtained:

    1 : Campylobacter pylori

    2 : Yersinia enterolytica type 3

    3 : Neisseria meningitidis

    4 : Neisseria gonorrhoeae

20   5 : Haemophilus influenzae type b

    6 : Kelbsiella pneumoniae

    7 : Salmonella typhimurium

    8 : Pseudomonas aeruginosa

    9 : Escherichia coli K1-083

25   HMW bacterial DNA and rRNAs could be detected using this procedure.

Section G: DNA/RNA purification of Escherichia coli JM101

    Isolation of NA from gram negative bacteria is
30 possible according to this invention. In bacterial cells high levels of high molecular weight DNA (HMW DNA) and ribosomal RNA are present. Example G1 shows that NA can be purified from bacterial cells using various chaotropic substances with silica as NA binding solid phase.

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Example G1: NA isolation/purification (endogeneous) from
bacterial cells with various chaotropic
substances and silica as NA binding solid
phase

NA was isolated from 50 μl overnight bacterial culture
JM101 in presence of 900 μl chaotropic substance and 40 μl
SiO_2. The high level of HMW-DNA and endogeneous ribosomal
RNA (16S and 23S) allows detection of isolated NA by UV
illumination of ethidium bromide stained gels. Isolations
were carried out according to protocol Y*, and 25% of the
eluted NA (40 μl portions) was analysed on agarose gel.

Table G1: Relative efficiency of HMW DNA and rRNA
isolation from bacterial cell samples using
various chaotropic substances in combination
with silica

Sample nr.	chaotropic substance used	relative efficiency of HMW-DNA recovery	relative efficiency of rRNA recovery
1	3M KI	1	>1
2	3M NaI	1	1
3	3M NaSCN	1	1

Legend:

Table G1 summarizes the results of the agarose gel
analysis. Quantification of HMW-DNA and rRNA recovery has
been compared with the procedure where GuSCN was used as
chaotropic substance in combination with silica: 1 in table
G1 indicates equally efficient DNA or RNA recovery. >1 in
table G1 indicates better recovery.

The E.Coli rRNA marker (Boehringer) was taken as a
reference for isolation endogeneous RNA from bacterial
cells.

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<u>Section H: DNA purification with alternative solid phase</u>
<u>capable to bind NA and guanidiniumthiocyanate</u>
<u>as chaotropic substance.</u>

5   To show that NA isolation/purification can be
performed with GuSCN and several silica derivates or
latexparticles (see material & methods) pure plasmid was
added in a low salt buffer (Tris 10 mM-EDTA 1 mM pH 8.0)
and then isolated according to protocol Y, however steps 7
10   and 9 were omitted (elution with TE was not carried out).
The silica/latex particles with bound NA were brought in
the PCR reaction mixture. The isolated DNA can be detected
by the PCR-method. Example H1 shows that NA an be purified
using alternative solid phases in combination with GuSCN as
15   chaotropic substance and detection by the PCR method.

<u>Example H1: DNA purification with alternative solid</u>
<u>phases and GuSCN</u>
         0.5 μg pGem3p24 present in 50 μl Tris 10 mM/EDTA 1 mM
20   pH 8.0 was mixed with 80 μl silica suspension or 80 μl
latex suspension (see Materials & Methods) and 900 μl lysis
buffer L6.
         After washing and drying at 56°C according to protocol
Y (no elution step) the pellet was resuspended in 50 μl
25   water. A 20 μl portion of the plasmid-silica suspension
was used in the PCR-mixture in presence of HIV specific
primers (Material & Methods), 5 μl of 10x concentrated
PCR-buffer, 1 μl 10 mM dNTPs, 2 Units <u>Taq</u> DNA polymerase
and water to a final volume of 50 μl were added and the
30   amplification reaction was started (1 cycle comprised 1
min. at 95°C; 1 min. at 37°C and 3 min. at 72°C).
         10 μl aliquots were taken from the reaction mixtures
after 30 cycles and analysed on a 2% agarose gel.

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Isolation of NA with the latex particles did not obtain
pellets like isolation of NA with silica.

When 1 ml washing liquid L2 was mixed with 300 μl 70%
EtOH a latex containing band was found between two liquid

5   phases.   The latex particles are detectable by their
colour. The latex containing fraction isolated was washed
twice with 70% EtOH and after centrifugation formed a small
pellet in the Eppendorff tube.

10   Table H1: Detection of DNA isolated using alternative
               solid phases in combination with guanidinium
               thiocyanate as chaotropic substance, using PCR
               amplification and gel analysis for detection.

sample nr.	NA solid phases	Detected level of HIV p24 DNA after amplification (LMW DNA)
1	Silica Coarse (control)	++
2	12 MAAM - C2	+
3	12 MAAM - C3	+
4	12 MAAM - C4	+
5	12 MAAM - C6	++
6	12 MAAM - C8	+
7	12 MAAM - C10	+
8	12 MAAM - C18	++
9	VQ 69 (Hydrophobic)	++
10	VQ 58B (Hydrophobic)	++
11	AGY 1515 (Hydrophilic)	+
12	AGY 27G (Hydrophilic)	+
13	ACN3red (Hydrophilic)	+

Legend:

The results are summarized in table H1. The expected
290bp HIV amplimer fragment was observed in all cases after
30 cycles. The size of the fragments was compared with

35   marker φx 174 RF DNA Hae III digest (Pharmacia) also loaded
on the gel.

++ : indicates the detection of the HIV specific 290 bp
     fragment on the agarose gel at an equal level as

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using Silica Coarse as solid phase (control).

+   : indicates a detectable level of the 290 bp
      fragment, lower than the control Silica Coarse.

5   Section I: Purification with NA-binding filters and
               GuSCN

      NA-binding filters (see Materials & Methods) can
replace the SiO_2 in the isolation of nucleic acid according
to protocol Y**.

10   Although normally no release of DNA takes place in the
low salt buffer (Tris 10 mM-EDTA 1mM pH 8.0) this optional
problem is set aside by inserting the filter with DNA bound
to it in the PCR-reaction mixture instead of eluting the
DNA from the filter. Example I1 shows that purification of

15 NA can be performed with a NA-binding filters and GuSCN as
a chaotropic substance analysed by the PCR-method.

    Example I1: DNA isolation/purification with a
               DNA-binding filter and detection by the

20   PCR-amplification

      Pure pGem3p24 DNA (concentration 1 μg; 0,01 μg and
0,005 μg) in 50 μl Tris 10mM/EDTA 1 mM pH 8.0 was added to
three DNA-binding filters, (PVDF, Hybond N and
Nitrocellulose) with a size of 1 cm x 1 cm and 900 μl GuSCN

25 (lysisbuffer L6).

      After washing (no centrifugation steps) and drying at
56°C (according to protocol Y**) the filter with DNA bound
to it was brought directly in the PCR-mixture. In presence
of HIV specific primers amplification was performed in the

30 PCR-cycler.

      The reaction mixture further consists of 5 μl 10 x
concentrated PCR-buffer, 1 μl 10mM dNTPs, 2 units Taq DNA
polymerase and water to a final volume of 50 μl.
Subsequently the amplification reaction was started.

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10 μl aliquots were taken from the reaction mixtures after 30 cycles (see example H1) and analysed on a 2% agarose gel.

5   Table I1: Detection of DNA isolated using filters as alternative NA binding solid phase in combination with GuSCN as chaotropic substance using PCR-amplification and gel analysis for detection.

sample nr.	NA binding solid phase	Amount of input DNA	Amount of HIVp24 DNA after amplification
1	Hybond N	1.0 μg	+
2	Hybond N	0.01 μg	o
3	Hybond N	0.005 μg	o
4	Nitrocellulose	1.0 μg	+
5	Nitrocellulose	0.01 μg	o
6	Nitrocellulose	0.005 μg	o
7	PVDF-millipore	1.0 μg	++
8	PVDF-millipore	0.01 μg	+
9	PVDF-millipore	0.005 μg	+

The result summarized in table I1. The expected 290 bp HIV amplimer fragment was observed. The fragment was compared with a commercial ϕxHaeII

25 ++: strong Ethidium bromide stained 290 bp fragment detectable on agarose gel

+: detectable 290 bp fragment

o: 290 bp fragment not detected

30 For comparison: 7ng of purified pGem3p24 DNA added to the PCR amplification mixture gives a 290 bp fragment quantified as ++

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Example   J: <u>Detection   of   RNA   from   Gram   Positive</u>
                 <u>Organisms</u>

It is well known that the cell walls of gram positive
microorganisms are much stronger than the cell walls of
5   gram negative microorganisms. We have found that treatment
with a chaotropic agent does not release the RNA of gram
positive   microorganisms   to   the   extent   needed   in
amplification technology. In order to overcome this
problem, while still maintaining the RNA released from
10   target cells in a condition in which an amplification
procedure can be performed, a pretreatment step is needed
before the procedure as described above can be done. This
is particularly important in the field of food diagnostics,
where human pathogens such as Listeria monocytogenes must
15   be   identified   prior   to   the   release   of   food   to   the
marketplace.

The pretreatment consists of taking a sample of food
or whatever substance is to be tested and mixing it with
lysozyme for a period long enough to partially degrade the
20   cell wall of the gram positive bacteria and then adding the
chaotropic substance, such as a lysing buffer as described
above and the nucleic acid binding solid phase, followed by
washing the solid phase and eluting to remove the bound
nucleic acid. It is believed that the pretreatment step
25   allows the gram positive organisms cell wall to become
fragile,   while   at   the   same   time,   gram   negative
microorganisms are totally lysed and their RNA degraded,
thereby removing much, if not all, non-specific RNA from
the sample tested. The chaotropic substance then lyses the
30   gram positive microorganisms, releasing RNA, and the method
continues as described previously.

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J1.   **Comparison of *L. monocytogenes* Nucleic Acid Extraction and Detection With and Without Lysozyme Pretreatment**

5   10^9 cfu/ml of *L. monocytogenes* was diluted 1000-fold to 10^6 cfu/ml. One set of 10^6 cfu/ml samples was prepared essentially via the procedure as described in Procedure Y*. A second set of 10^6 cfu/ml samples were treated with 1.2 mg/ml of lysozyme from egg whites (bought from Sigma
10   Chemical Company, St. Louis, Missouri, USA) for 20 minutes at 37°C by adding $3\mu l$ of a 40 mg/ml lysozyme stock solution to a *L. monocytogenes* sample resuspended in 100 μl of 50 mM Tris pH 7.6, 6.7% sucrose. Subsequently, 1 ml of L6 buffer and 50 μl silica were added and the nucleic acid was
15   isolated as described in Procedure Y*.

An aliquot of nucleic acid was removed for NASBA amplification using a 16S rRNA *L. monocytogenes* target sequence. Briefly, primer 1 contains the T7 RNA polymerase recognition site attached to nucleotides 1493-1475 in 16s
20   rRNA and primer 2 contains the T7 RNA polymerase recognition site attached to nucleotide 1221-1239 of 16s rRNA. The resulting 272 bp amplified product was hybridized to a 16S rRNA detection probe using the Gen-Probe *L. monocytogenes* culture identification kit. This
25   kit uses a chemiluminescent labeled, single-stranded DNA probe that is complementary to the rRNA of *L. monocytogenes*. After the rRNA is released from the organism, the labeled DNA probe combines with the target organisms rRNA to form a stable DNA:RNA hybrid. The
30   Selection Reagent allows for the differentiation of non-hybridized and hybridized probe. The labeled hybrids are measured in the Gen-Probe luminometer.

The Gen-Probe Pal-Accu LDR™ machine was used to detect the NASBA amplification product. Greater than 1,500 Pal

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units was indicative of a positive result and less than
1,500 Pal units, a negative result. This experiment was
performed in duplicate on two separate days. The results
are:

5   Table J1.

Sample	Pal	Pal
10^6 cfu: with lysozyme	5162 (+)	7864 (+)
10^6 cfu: without lysozyme	928 (-)	1268 (-)

10   These data indicate that 10^6 cfu/ml can be detected
only if the lysozyme pretreatment step is utilized in pure
culture.

         J2.   **Comparison of Lysozyme Pretreatment with**
15   **Heating L6 Buffer in Pure *L. monocytogenes***
                      Cultures

         10^9 cfu/ml of pure *L. monocytogenes* was subjected
either to lysozyme pretreatment as described in example J1,
20 or to directly heating at 95° or 100°C in the L6 buffer.
The RNA was diluted 100,000-fold and this sample, 10^4
cfu/ml, was amplified by NASBA and detected. The results
are described in the following table.

25   Table J2.

Sample	Treatment	Pal
Lysozyme Pretreatment	800 µg/ml, 37°C, 20 minutes	7929 (+)
L6 Buffer	95°C, 5 minutes	6398 (+)
L6 Buffer	95°C, 10 minutes	2632 (+)
L6 Buffer	100°C, 2 minutes	7527 (+)
L6 Buffer	100°C, 5 minutes	4713 (+)

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This experiment indicates that with pure *L. monocytogenes* cultures containing 10^4 cfu/ml, heating the L6 buffer can result in amplifiable and detectable 16s rRNA. The PAL levels are either similar to or considerably lower than those seen with lysozyme pretreatment step. However, as shown in example J3, heating the L6 buffer is not nearly as efficient a methodology when food matrix is present with the *L. monocytogenes* culture.

10   J3.   **Comparison of Lysozyme Pretreatment with Heating L6 Buffer in Shrimp Cultures**

An overnight culture of *L. monocytogenes* of 10^9 cfu/ml was diluted either 100,000 or 10,000-fold to either 10^7 or 10^5 cfu/ml. 25 grams of raw shrimp was grown for 24 hours in Frasers medium, and 1 ml of this shrimp culture was mixed into either 10^7 or 10^5 cfu/ml of *L. monocytogenes*. This mixture was then processed either by A) lysozyme pretreatment or B) heating with L6 buffer at 95°C for 1.5, 2, or 3 minutes. The results are as follows:

Table J3.

Sample	Pretreatment	L. mono cfu	Shrimp	PAL
1	Lysozyme	10^7	None	9466 (+)
2	Lysozyme	10^5	None	9212 (+)
3	Lysozyme	10^7	1 ml	9262 (+)
4	Lysozyme	10^5	1 ml	5689 (+)
5	95°C, 1.5 min	10^7	1 ml	7081 (+)
6	95°C, 1.5 min	10^5	1 ml	793 (-)
7	95°C, 2 min	10^7	1 ml	4579 (+)
8	95°C, 2 min	10^5	1 ml	1026 (-)
9	95°C, 3 min	10^7	1 ml	5300 (+)
10	95°C, 3 min	10^5	1 ml	901 (-)

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    While the presence of shrimp culture slightly mitigates the strongly positive signal when lysozyme pretreatment is used (compare Pal units for sample 4, versus sample 2), a clear distinction between lysozyme
5   pretreatment and L6, 95°C heating, is noted when shrimp is present. These results indicate that heating the L6 buffer at 95°C for 1.5, 2 or 3 minutes is significantly inferior to lysozyme pretreatment as 10^5 cfu/ml is strongly positive when lysozyme pretreatment isolation is utilized (sample 4,
10   5689 Pal). However, the equivalent 10^5 cfu/ml heated at 95°C and processed without lysozyme were definitively negative (samples 6, 8 and 10: 793, 1026 and 901 Pal units). This example clearly indicates that while in previous Example J2, heating the L6 buffer may be partly
15   amenable for pure *L. monocytogenes* cultures, in shrimp culture, the lysozyme pretreatment step is necessary.

        J4.   **Optimization of Lysozyme Pretreatment at Ambient Temperature**

20

    800, 1,600, 2,400 or 3,200 µg/ml of lysozyme was added, for 20 minutes, at ambient temperature (20 to 23°C ±1°), to 10^9 cfu/ml of *L. monocytogenes* to determine optimal concentration of lysozyme at this temperature. RNA
25   was subsequently processed as described above. Total RNA was resolved on 1.2% agarose/formaldehyde containing gels and *L. monocytogenes* 16s rRNA bands were visually inspected. The 1,600 µg/ml pretreatment at ambient temperature clearly contains the highest yield of nucleic
30   acid, as shown in Figure 1.

    Therefore, the lysozyme pretreatment can occur over a range of temperatures, from 0°C to about 40°C, providing an acceptable amount of nucleic acid available for amplification.

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Claims

1. A process to amplify captured nucleic acid from a
   gram positive microorganism in a starting material,
5 comprising mixing the starting material with a
   lysozyme, then adding a chaotropic substance and a
   nucleic acid binding solid phase, separating the
   solid phase with the nucleic acid bound thereto from
   the liquid, washing the solid phase-nucleic acid
10 complexes, and contacting the solid phase-nucleic
   acid complexes with a mixture comprising components
   that amplify the nucleic acid.

2. A process for isolating nucleic acid from a gram
   positive microorganism in a starting material,
15 comprising mixing the starting material with a
   lysozyme, then adding a chaotropic substance and a
   nucleic acid binding solid phase, separating the
   solid phase with the nucleic acid bound thereto from
   the liquid, and washing the solid phase nucleic acid
20 complexes.

3. A process according to claims 1 or 2, wherein the
   starting material is food.

4. A process according to claims 1 or 2, wherein the
   lysozyme is mixed with the starting material for
25 about 20 minutes at a temperature not more than 40°
   C.

5. A process according to claims 1 or 2, wherein the
   lysozyme is mixed with the starting material for
   about 20 minutes at room temperature.

30 6. A process according to claims 1 or 2, wherein the
   gram positive microorganism is a _Listeria_ species.

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2λ 4λ 6λ 8λ
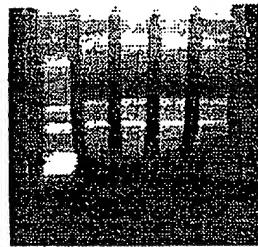
μy LYSOZYME

.80 .160 .240 .320
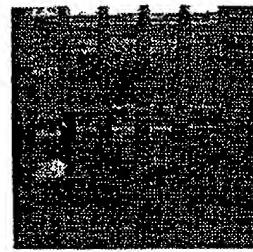
FIG. 1

In tional Application No

PCT/US 94/08638

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/10 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 389 063 (AKZO N.V.) 26 September 1990 the whole document	1-6
Y	INFECTION AND IMMUNITY, vol.44, no.1, April 1984, AM. SOC. MICROBIOL., BALTIMORE, US; pages 157 - 161 R.K. FLAMM ET AL. 'Introduction of pAMbeta1 into Listeria monocytogenes by conjugation and homology between native L. monocytogenes plasmids' see page 158, left column, line 12 - line 25	1-6

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 October 1994	08 -11- 1994

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 314 294 (GENE-TRAK SYSTEMS) 3 May 1989 see page 6, column 10, line 54 - page 7, column 11, line 4 ---	1-6
Y	WO,A,90 08841 (GENE-TRAK SYSTEMS) 9 August 1990 see page 19, line 23 - line 28 ---	1-6
Y	EP,A,0 261 955 (E.I. DU PONT DE NEMOURS AND COMPANY) 30 March 1988 see page 3, line 40 see page 5, line 34 ---	1-6
Y	EP,A,0 261 956 (E.I. DU PONT DE NEMOURS AND COMPANY) 30 March 1988 see page 2, column 1, line 32 - line 43 -----	1-6

1

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0389063	26-09-90	NL-A-	8900725	16-10-90
		AU-B-	641641	30-09-93
		AU-A-	5215390	27-09-90
		CA-A-	2012777	23-09-90
		JP-A-	2289596	29-11-90
		US-A-	5234809	10-08-93
EP-A-0314294	03-05-89	US-A-	5089386	18-02-92
		AT-T-	110419	15-09-94
		AU-A-	2213488	16-03-89
		DE-D-	3851200	29-09-94
		JP-A-	1304899	08-12-89
WO-A-9008841	09-08-90	AU-A-	5188190	24-08-90
		CA-A-	2025236	07-08-90
		EP-A-	0418346	27-03-91
		JP-T-	3504677	17-10-91
EP-A-0261955	30-03-88	JP-A-	63098400	28-04-88
EP-A-0261956	30-03-88	US-A-	4900677	13-02-90
		JP-A-	63091093	21-04-88